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APPLICATION NUMBER: *60/425,228*
FILING DATE: *November 08, 2002*
RELATED PCT APPLICATION NUMBER: *PCT/US03/35734*

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11-12-02

PTO/SB/16 (10-01)
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This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EV084123770US

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☐ Additional inventors are being named on the _____ separately numbered sheets attached hereto

TITLE OF THE INVENTION (500 characters max)

Expanding Human Embryonic Stem Cells Using Human Adult(Marrow) Cells or Derived Molecules

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ Specification *Number of Pages* 47
☑ Drawing(s) *Number of Sheets* 11
☐ Application Data Sheet. See 37 CFR 1.76
☐ CD(s), Number
☐ Other (specify)

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☑ Applicant claims small entity status. See 37 CFR 1.27.
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FILING FEE AMOUNT ($): $80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ No.
☒ Yes, the name of the U.S. Government agency and the Government contract number are: CA06973

Respectfully submitted,

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Date 11-08-02

REGISTRATION NO. *(if appropriate)* 45,282

Docket Number: DM-4064

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U.S. Provisional Patent Application

JHU Ref. No. DM-4064

Expanding Human Embryonic Stem Cells Using Human Adult (Marrow) Cells or Derived Molecules

Inventor: Linzhao Cheng

DM-4064

Running title: Growth of human embryonic stem cells on human feeder cells

Human adult marrow cells support prolonged expansion of human embryonic stem cells in culture

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Funding: This work was supported in part by research grants from the WW Smith Charitable Trust and National Institutes of Health.

Contents of this manuscript were included in an abstract submitted to the American Society of Hematology (ASH) 44th annual meeting. The abstract is accepted as an oral presentation scheduled on December 9, 2002 during the ASH meeting.

Key Words: embryonic stem cells; mesenchymal stem cells; marrow stromal (stem) cells; self-renewal; SSEA-4; Oct-4

Introduction

Embryonic stem (ES) cells are continuous proliferating stem cell lines of embryonic origin first isolated from the inner cell mass (ICM) of mouse blastocysts 20 years ago. The distinguishing features of these ES cells (*versus* committed stem cells found in adults) are their capacity to be maintained in an undifferentiated state infinitely in culture and their potential to develop into every cell of the body. Based on previous methods developed for mouse ES cells, Dr. James Thomson first reported a method to establish human ES cell lines (1). Like mouse ES cells, human ES (hES) cells can proliferate in culture for years and maintain a normal karyotype. Both mouse and human ES cells express high levels of a membrane alkaline phosphatase (APase) and Oct-4, a transcriptional factor critical to ICM and germline formation (1-5). Unlike mouse ES cells, hES cells do not express the stage-specific embryonic antigen (SSEA)-1, but express SSEA-4 which is another glycolipid cell surface antigen recognized by a specific monoclonal antibody (1-5). The growth requirements of hES cells are also different. Prolonged propagation of hES cells is typically achieved by co-culture with primary mouse embryonic fibroblasts (MEFs) serving as feeder cells. The existing hES cell lines were not able to maintain their undifferentiated state in the absence of supporting feeder layer cells, even when exogenous cytokines such as LIF and gelatin-coated plates are used (1, 3, 4). Differentiated hES cell colonies (formed either in the absence of feeder cells or after extended culture without appropriate splitting) gradually lose the SSEA-4 and Oct-4 expression (2, 5-6). Xu et al. and colleagues reported recently that viable MEFs may not be necessary and hES cells may be maintained on extracellular matrix (ECM) if the conditioned medium from MEFs is provided (7). The ECM used in this study is Matrigel, which is the trademark of a crude extract of basement membrane matrices from mouse sarcomas. It is unclear that the above feeder-free culture method can actually *expand* (net increase) or merely *maintain* undifferentiated hES cells in culture for the reported culture period. Nonetheless, the use of uncharacterized rodent cells such as MEFs or

rodent tumor crude extracts makes cultured hES cells xenogenic biologics and imposes an extra risk to the clinical utility of hES cell lines (4).

In adults, several distinct types of pluripotent stem cells have been isolated from BM. Adult BM is the primary site of hematopoietic stem cells (HSCs), the common precursor of blood and immune cells. Beginning with pioneering work by Friedenstein more than 30 years ago, it is well recognized that non-hematopoietic stem cells are also present in bone marrow of adult humans and animals (8-9). The most characterized type is mesenchymal stem cells (MSCs) capable of generating mesenchymal cells and stromal cells that support hematopoiesis (10-15). It was also reported in recent years that certain freshly isolated or culture expanded BM cells can differentiate into many other types of cells such as hepatocytes in liver, neurons and glial cells in brain, satellite cells in skeletal muscles and cardiomycytes in the heart (16-24). Thus, adult BM contains cells and microenvironment that are able to maintain stem cells in their undifferentiated states. These studies led us to investigate whether adult BM-derived cells can also support the growth of hES cells.

In recent years, several groups have developed improved methods to obtain large numbers of marrow stromal progenitor cells in culture from adult human BM aspirates, either by physical enrichment of precursor cells followed by culture expansion, or by direct culture selection and amplification. These marrow fibroblastic cells have been termed as either stromal progenitor cells reflecting their proliferation potential in culture (11), marrow stromal cells (MSCs) reflecting the source and method of the derivation (12-14), or mesenchymal stem cells (MSCs) reflecting their proven potentials to generate multiple types of mesenchymal cells when exposed to appropriate stimuli in vivo or in vitro (10, 25). The latter two methods, which are very similar in practice, are widely used by many investigators including ourselves

(26). These marrow-derived (fibroblastic) stromal cells that function as non-hematopoietic multipotent stem cells are collectively called herein as MSCs. After two passages (approximately 14 cell divisions) in a selective medium supplemented with fetal bovine serum (FBS), culture-expanded human MSCs (hMSCs) are morphologically and phenotypically homogenous and essentially free of endothelial cells, macrophage or adipocyte contamination (25-26).

These culture expanded and highly homogenous hMSCs enable us to perform detailed analyses which were previously impossible with mixed "stromal" cell populations. When used as adherent feeder cells, the culture-expanded hMSCs supported human $CD34^+$ HSCs in long-term culture assays and their differentiation into erythroid, myeloid, megakaryocytic, osteoclastic or B cell lineages even in the absence of added cytokines (26-31). The activity is due to, at least in part, the production of various hematopoietic cytokines including LIF, IL-6, IL-11 as well as SCF and Flt3/Flk2 ligand (FL) by hMSCs (26-28, 31). Thus, our goal of the present study was to investigate whether culture-expanded hMSCs derived from human adult BM can also support the growth of hES cells in a culture medium that is known to favor their proliferation while retain the undifferentiated state.

We report here that culture-expanded MSCs can replace MEFs and fully support prolonged expansion of hES cells in culture. Human ES cells co-cultured on irradiated hMSCs expanded >100 fold during the 30 day continuous culture (in 5 passages). The expanded hES cells after 9 passages maintained their normal karyotype. Moreover, the expanded hES cells retained unique hES cell morphology and expressed markers such as APase and SSEA-4 characteristic of undifferentiated hES cells.

Materials and Methods

Human MSC Isolation and expansion. BM samples collected from healthy and consented human donors were purchased from AllCells company (Berkeley, CA). The use of anonymous primary human cells for laboratory research was approved by the Internal Review Board of the Johns Hopkins medical Institutions. Mononuclear cells (MNCs) were isolated from heparinized BM aspirates (diluted with equal volume of phosphate buffered saline) by the standard density (1.077 g/ml) centrifugation using Ficoll (Pharmacia, Piscataway, NJ). As compared to a previous protocol (25-26) using Percoll (1.073 g/ml, also from Pharmacia), the Ficoll method yielded 2 fold more MNCs but generated the same total numbers of MSCs after culture expansion per unit volume of BM samples. MNCs at the interface were recovered, washed and finally resuspended in the hMSC medium composed of Dulbecco's Modified Eagles Medium (DMEM) with low glucose (Invitrogen, Carlsbad, CA), 10% FBS, 1% antibiotic-antimycotic stock solution (Invitrogen) as previously described (25-26), in the absence or presence of 1 ng/ml basic fibroblast growth factor (bFGF, Invitrogen). The addition of bFGF to the "generic" hMSC medium gave consistent and optimal growth with different FBS batches from various suppliers (Hyclone Laboratories, Logan, UT, Invitrogen or Gemini, Calabasas, CA). For Primary cultures, cells were plated into 175 cm^2 flasks at a density of $6x10^7$ MNCs/flask and the cultures were incubated at 37°C in 5% CO_2 in air and 95% humidity. The medium was exchanged after 48 hours and every 3-4 days thereafter. When cells in the primary passage reached approximately 90% of confluence (in approximately 2 weeks), hMSCs were recovered by digestion with 0.05% trypsin/0.53 mM EDTA solution (Invitrogen) and re-plated into passage culture at a density of 5,000 to 10,000 cells per cm^2. Once confluent (10-14 days), harvested

(passage 1) cells were seeded similarly to obtain passage 2 (p2) cells and so on. For the colony formation assay, aliquots (up to 1.6 x10^6 cells/well) of MNCs were plated into 6-well culture dishes for 14 days with the complete MSC medium and numerated as described before (26). Fibroblastic MSC colonies were found in MNCs from bone marrow (approximately one in 10,000 and 20,000 ? MNCs isolated by the Percoll method, respectively), but <1 per 6x10^7 MNCs isolated from cord blood or peripheral blood with or without the G-CSF mobilization.

Human ES cells. The H1 (also known as WA01 in the NIH Embryonic Stem Cell Registry) hES cell line (p22) was obtained from the WiCell Research Institute (Wisconsin, MI), and initially cultured as instructed by the provider. MEFs (p3) purchased from Specialty Media, Inc. (Phillipsburg, NJ, www.specialtymedia.com) were used as feeder cells for the hES cells. MEFs or MSCs (200,000 cells) were plated per well (9.4 cm^2) in 6-well plates after being irradiated (50 Gy, 1 Gy = 100 rads) using a ^{137}Cs gamma-irradiator. The hES cell culture medium consists of 80% (v/v) KO-DMEM, 20% (v/v) of the KO Serum Replacement, 2 mM Glutamine, 10 mM non-essential amino acids, 50 μM β-Mercaptoethanol and 4 ng/ml bFGF (Invitrogen). Cell cultures were incubated at 37°C in 5% CO_2 in air and 95% humidity. Once hES cell colonies grew maximal sizes before the onset of visible differentiation, cells in the co-culture (hES cells and irradiated MEFs) were digested and seeded onto freshly-prepared feeder cells. Initially we used collagenase IV (1 mg/ml) to split cells (1:1 to 1:3) as instructed and published previously (1, 3). Later we harvested cells by digesting cells in co-cultures with 0.05% trypsin/0.53 mM EDTA solution for 5 min. The digestion was stopped by adding soybean trypsin inhibitor (Sigma, St. Louis, MO). After wash, the dissociated cells in the hES cell culture medium were

split from 1:1 to 1:50, and seeded onto feeder cells or 6-well plates coated with diluted (1/20) Matrigel (Becton Dickson Labware, Bedford, MA) as described previously (7).

Immuno-fluorescence and APase staining. Co-cultures used for APase staining or immuno-fluorescence analysis were established in either 6-well or 24-well plates. Prior to analysis, adherent cell layers were fixed by the addition of 10% formalin (15 minutes). After wash with a Tris-based saline solution, APase staining was performed using a kit containing BCIP/NBT as the substrate (Sigma). The dark blue staining was visualized by light microscopy. The fixed cells in co-cultures were also stained with mouse monoclonal antibodies (mAbs) against either SSEA-4 (clone MC-813-70, isotype IgG3) or SSEA-1 (clone MC-480, isotype IgM). Hybridoma supernatants of both mAbs were obtained from Developmental Studies Hybridoma Bank (Iowa City, IA). For the immunofluorescence staining, the fixed cells were incubated 15 minutes with goat serum (~~a~~ 2% ~~solution~~) to block non-specific binding. The co-cultures were stained with diluted (1:100) hybridoma supernatants recognizing either SSEA-4 or SSEA-1 antigen. After incubation in the dark for 1 hour at 25°C or overnight at 4°C, fixed cells were washed extensively before the secondary staining reagent was added. Goat anti-mouse IgG conjugated to the fluorochrome Alexa 546 (Molecular Probes, Portland, Oregon) was added for 45 minutes at 25°C. The nuclei of hES cells and hMSCs were counter-stained by Hochest 33358 (Molecular Probes). Immunofluorescence analysis was performed with a Nikon (TE300) microscope with separate filters for either Hochest (blue) or red fluorescence, or a triple filter for blue, green and red fluorescence simultaneously. The fluorescence and light images were recorded in Kodak film (ASA400). The scanned image was analyzed by Photoshop 4.0.

Cell isolation by magnetic cell sorting (MACS). Cells were harvested from co-cultures by gentle digestion with 0.25% Trypsin/0.53 mM EDTA solution and washed once by PBS containing 2% BSA and 2 mM EDTA. Before incubating with the SSEA-4 mAb (1:100), cells were pre-incubated with human IgG (2 mg/ml) to block non-specific IgG binding. The SSEA-4 labeled cells were incubated with magnetic beads conjugated with anti-mouse IgG antibodies (Miltenyi Biotec, Auburn, CA). The labeled cells were isolated by the miniMACS magnet stand and the large cell isolation column as instructed (Miltenyi Biotec).

Flow cytometric analysis. Cells were harvested as described above and suspended in 100 µl staining buffer (2% BSA, 2 mM EDTA and 0.1% sodium azide in PBS) containing human IgG to block non-specific IgG binding. The diluted (1:100) SSEA-1 or SSEA-4 mAbs were added as primary antibodies. FITC or R-phycoerythrin (PE) conjugated anti-mouse IgG antibodies were used to detect binding of the SSEA-4 mAb (mouse IgG3). For the SSEA-1, anti-mouse IgM antibodies conjugated with PE were used. These secondary reagents were purchased from Caltag (Burlingame, CA) or Becton Dickinson/ PharMingen (San Jose, CA). In addition, we also used PE conjugated mAbs recognizing SH-2/endoglin/CD105 (clone SN6 or 266), HLA-ABC/MHC class I (clone TU149), HLA-DR/MHC class II (clone L233), CD133 (clone AC133-1), Thy-1/CD90 (clone F15-42-1-5), CD34 (Clone HPCA-2) and PECAM-1/CD31 (MBC78.2), in conjunction with SSEA-4 and the FITC conjugated anti-mouse IgG antibody (for hES cells). These directly PE conjugated mAbs were purchased from Caltag, Miltenyi Biotec, Beckman Coulter (Miami, FL) and Becton Dickinson/ PharMingen (San Jose, CA), and used as instructed by providers. A FACScan® flow cytometer (Becton Dickinson) was used for these analyses.

Ten thousand events were acquired for each sample and analyzed using the CellQuest software (Becton Dickinson).

Karyotypic analyses of hES cells. Before and after co-cultures on hMSCs, karyotyping analyses of hMSCs or hES cells were carried out by the Laboratory of Prenatal and Research Cytogenetics in Department of Ob/Gyn at the Johns Hopkins Hospital. The method is essentially the same as previously described (32). Briefly, cells in co-culture were incubated with 0.1 μg/ml of Colcemid for 3-4 hr, trypsinized, resuspended in 0.075 M KCl, incubated for 20 min at 37°C, and then fixed in 3:1 methanol/acetic acid. After staining, karyotypes of normal human chromosomes were examined by cytogenetics specialists at the 300-band level of resolution.

RNA preparation and gene expression analysis. Using the RNeasy kit (Qiagen, Valencia, CA), total RNA was extracted from MACS-isolated hES cells that have been cultured on MEFs or hMSCs, or from control MEFs or hMSCs cultured in the same hES cell culture medium. The contaminating genomic DNA was further eliminated by DNase I digestion. The first strand cDNA synthesis was performed using the Superscript II reverse transcriptase (RT) and oligo$(dT)_{12\text{-}18}$ as primers (Invitrogen). Aliquots (10%) of the RT product were used as a template for PCR amplification with specific primer sets for either human Oct-4 or human/mouse β-actin gene. The oligonucleotide primer pairs used for Oct-4 RT-PCR were based on a previously report (6): Oct-4 sense, 5'-CGTGAAGCTGGAGAAGGAGAAGCTG-3'; Oct-4 antisense, 5'-CAAGGGCCGCAGCTTACACATGTTC-3'. The two primers (nt. 862-886 and nt. 4527-4551 in the Oct-4 genomic DNA accession Z11900) are located in two different exons. The detected cDNA fragment by RT-PCR was 140 bp long as predicted. The primers for the β-actin gene are Actin–sense: 5'-GCTCGTCGTCGACAACGGCTC-3'; Actin-antisense: 5'-CAAACATGATCTGGGTCATCTTCTC-3'.

The RT-PCR product of human and mouse β-actin cDNA is 353 bp long. After 40 cycles of PCR with an annealing temperature at 60°C, the RT-PCR products were visualized by ethidium bromide staining, following electrophoresis through a 1.5% agarose gel.

RESULTS

The origin and growth of hES cells

We obtained the H1 hES cell line from the WiCell Research Institute and expanded the stock in co-culture on irradiated (mitotically inactive) MEFs as instructed by the provider, similar to previous publications (3, 5, 33). We continuously cultured these ES cells for 3 months and split (using collagenase IV) approximately once a week. Consistent with the provided protocol, we observed ~2 fold expansion every passage (~ 7 days). Once enough cells were obtained, we attempted to improve new culture conditions and different splitting (passaging) methods. We observed consistently that splitting by trypsin/EDTA digestion resulted in more uniform and greater numbers of hES cell colonies in subsequent culture than the collagenase method. Thus, we chose to use the trypsin/EDTA digestion method to maintain and expand hES cells co-cultured on either MEFs or hMSCs described below.

An improved method to derive and expand hMSCs

The method we used to derive and expand hMSCs from adult BM is similar to that previously described (25-26) with a few modifications including supplementing the culture medium containing 10%FBS with bFGF (1 ng/ml). Consistent with an early report (34), we found that adding the low concentration of bFGF provides a consistently optimal growth condition and essentially alleviates the need to screen favorable FBS lots. Using this improved method, we can efficiently and consistently derive and expand hMSCs from multiple male and female donors. After a primary and 2 subsequent passages in culture (a total of ≤6 weeks), 75-200 million (p2) hMSCs can be obtained from ~100 x 10^6 MNCs in a 10cc BM aspirate sample. Similar to the previous method, the expanded hMSCs were highly

uniform in morphology and phenotypes, essentially free of adipocytes, hematopoietic cells ($CD45^+$) and endothelial cells ($CD34^+$ or $CD31^+$). As previously described (25, 31), the expanded hMSCs in culture expressed unique markers such as CD105 (also known as SH-2 and endoglin) and Thy-1/CD90 (Table 1).

Prolonged expansion of hES cells co-cultured on hMSCs

We observed that undifferentiated hES cell colonies also formed on hMSC feeders in the hES cell (serum-free) culture medium, despite that their growth rate was initially lower than that on MEFs. We continuously cultured hES cells on irradiated or non-irradiated hMSCs for additional 4 passages (~ 4 weeks, with split ratios from 1:2 to 1:5 in each passage), and then characterized resulting hES cells in detail. Morphology of hES cell colonies expanded on MSCs are shown in Fig 1. In the absence of seeded hES cells (i.e. hMSCs alone), no hES cell-like colonies formed with or without irradiation (not shown). On non-irradiated hMSCs, hES cell colonies had a better growth rate and more compact morphology (Fig 1A). However, the proliferation of hMSCs in co-culture imposed a practical difficulty, i.e., overgrowth of hMSC feeder cells that divided faster than hES cells. Subsequently, we discontinued the co-culture of hES cells with proliferating MSCs and used exclusively irradiated MSCs as feeder cells. On irradiated hMSCs, hES cell colonies amplified >100 fold during the 30 day continuous culture (in 5 passages). In multiple experiments we performed so far, the longest continuous co-culture of hES cells with irradiated MSCs is 13 passages.

We attempted to compare directly the growth of hES cells on hMSCs with the growth on MEFs as well as on different preparations of hMSCs from various donors. A 1:20 split of p6 hES cells on MSCs (#1) was seeded onto duplicate wells of irradiated MEFs or MSCs from two donors (#1 and #2). Six

days after, numbers of hES cell colonies (≥50 cells) with the undifferentiated morphology were counted (Fig 2). Both MSCs feeders gave rise to similar numbers of hES cell colonies. We estimated that we had ≥5 fold expansion in this passage. So far, different preparations (p2 to p5) of hMSCs from 3 donors (two males and one female) have been used, and they gave similar results (not shown). Under the same culture condition, Matrigel was insufficient to support hES cell growth, regardless whether the conditioned medium from MSCs or MEFs was provided (not shown). The MEF feeder gave rise to fewer and smaller ES cell colonies (~ 2 fold expansion). Therefore, we found that hES cells appeared to grow better on hMSCs than MEFs, once they adapted on hMSCs (6 passages in this experiment). This result suggest an "adaptation" hypothesis which also explains the fact that hES cells grew poorer initially on hMSCs when passaged initially from the co-culture with MEFs.

The expanded hES cells co-cultured with hMSCs retained molecular markers unique to undifferentiated hES cells.

After 4 or more passages on hMSCs, aliquots of the expanded hES cells were analyzed regarding the expression of cell-surface markers such as APase and SSEA-4. The APase isoform on ES cells is likely to be EC.3.1.3.1, which is also known as the liver/kidney/bone APase or the tissue non-specific APase (5), and sensitive to levamisol inhibition. By histochemical staining, hES cells colonies were strongly APase positive, while hMSCs as feeder cells were largely negative (Fig 1C). APase activities were preferentially expressed on cell membrane of hES cells and sensitive to levamisol inhibition (not shown). Like irradiated MEFs, few singular MSCs with apoptotic morphology (broad and flat) displayed a weak APase activity (not shown). The absence or low level of APase activities on the viable MSCs is

consistent with the report that undifferentiated hMSCs are APase negative until induced to differentiate to osteoblasts (35). The expanded hES cells were also stained for SSEA-4, a glycolipid antigen expressed on hES cells but not mouse ES cells. After the fixation, the co-cultures of hES cells with irradiated hMSCs (p4) were stained with or without a mouse mAb against the SSEA-4 antigen (Fig 3). A high level of SSEA-4 expression was found in expanded hES cells while absent in the hMSC feeder cells (A and C).

We have also employed the mAb against the SSEA-4 surface antigen and MACS to isolate live undifferentiated hES cells that expressed SSEA-4 (SSEA-4$^+$) after c-culture on MSC or MEF feeder cells. Cells in co-cultures were digested to single cell suspension and pooled for each group. The labeled SSEA-4$^+$ cells were then isolated by the Miltenyi's MACS system. Cells were also analyzed by flow cytometry before or after the MACS isolation (Fig 4). Flow cytometric analysis further confirmed that The hES cell fraction (9.6% in Fig 4A) retained a high level of the SSEA-4 expression. After the MACS isolation, the purity of hES cells (SSEA-4$^+$) was typically ~95%. The isolation method allowed us to obtain a hES cell population essentially free of feeder cells.

With these highly purified hES cells that had been cultured on either hMSCs or MEFs (as shown in Fig 2), we analyzed the gene expression of Oc-4, a critical transcription factor that is known to be preferentially expressed in undifferentiated hES cells (1-3, 5). By RT-PCR (Fig 5), a high level of the Oct-4 expression was detected in the hES cells cultured either on MEFs (lane 1) as described previously. We also found that hES cells that had been cultured on hMSCs for 5 passages also expressed the Oct-4 gene at a high level (Lane 3). In contrast, hMSCs alone expressed a very low but detectable level of the Oct-4 gene (Lane 4).

In addition, we analyzed the expression of other cell-surface markers on hES cells cultured on MSCs, or MSCs alone by flow cytometric analysis as in Fig 4. The result is summarized in Table 1. The expression pattern of these unique, yet not definite markers, is consistent with previously reports with the H1 and several other hES cell lines (5, 33, 36). Based on their morphology, the Oct-4 gene RT-PCR analysis and the expression of 11 unique cell-surface markers including APase and SSEA-4, we conclude that the hES cells expanded by co-culture on MSCs retained a unique morphology and phenotype characteristic of undifferentiated hES cells as did on MEFs.

Expanded hES cells on MSCs have a normal chromosomal karyotype

In one continuous co-culture on irradiated hMSCs that lasted for 9 passages, we attempted to examine chromosomal karyotypes of the expanded hES cells. Before the expansion, we had confirmed the H1 ES cell line (which was derived from a male embryo) retained a normal 44+XY karyotype after continuous expansion on MEFs for 3 months in our laboratory (not shown). To easily identify hES cells and distinguish them from hMSCs that were also present in co-cultures, we needed to reduce numbers of human feeder cells, particularly those of the male hMSCs that we used in the first 7 passages. To this end, we seeded hES cells onto female hMSC feeder ecll for two more passages followed by two additional passages on MEFs. Karyotyping analyses were performed by the Laboratory of Prenatal and Research Cytogenetics at Johns Hopkins Hospital. Fig 6 shows a representative readout of such chromosomal karyotyping. Out of 5 samples examined, all of them displayed a normal 44+XY chromosomal karyotype as the original. Thus, hES cells retained a stable and normal karyotype after prolonged expansion on hMSCs.

Discussion

Expanding hES cells efficiently under a clinically applicable culture condition is a prerequisite for their use in novel cell and gene therapies. We report here that we discovered a method to expand hES cells with the use of adult human cells derived from adult BM. We developed an improved method to expand hMSCs and used them to support prolonged growth of hES cells. Irradiated MSCs from various donors at passage 2-5 supported the hES cell expansion in a serum-free medium at a rate similar to MEFs. The amplified hES cells on hMSCs displayed the unique morphology and molecular markers characteristic of undifferentiated hES cells as did on MEFs, and retained a normal chromosomal karyotype.

Currently we do not know the identity of molecules that are made by hMSCs or MEFs and required by hES cells in culture. In Fig 2, we found that Matrigel (even with the conditioned medium from hMSCs or MEFs) was insufficient to maintain hES cells. This result indicated that currently we could not separate soluble factors made by hMSCs from required ECM and then reconstitute a feeder cell-free culture system. Our result and a recent report (37) contradicted to a previous report by Xu et al. (7) that hES cells could be maintained in an undifferentiated state if they were cultured on Matrigel as ECM together with (undiluted) conditioned medium from MEFs. The apparent discrepancy may be due to varied levels of "carryover" MEFs in the Matrigel "feeder cell-free" culture. Xu et al. did not use purified hES cells free of MEFs also present in co-cultures, and did not address the level and importance of the carryover MEFs in their Matrigel "feeder cell-free" culture (7). Since the majority of irradiated MEFs in hES cell co-culture survived for at least 2 weeks, the "carryover" MEFs from the previous passage, although reduced in numbers, were also present in the Matrigel culture for several passages. For

[redacted]

example, when an aliquot of hES cell co-cultures (e.g., 1:3) was plated on Matrigel-coated plates, the equal fraction of MEFs (~60,000) were also seeded simultaneously in the new culture. Using the described method by Xu et al., indeed we could also maintain or moderately (~2 fold) expand hES cell colonies in the subsequent passage, if the splitting ratio of co-cultures was 1:1 to 1:3. However, we were unable to maintain the majority of hES cells in an undifferentiated state on Matrigel after 4-5 passages (~30-40 days, when numbers of remaining MEFs were negligible) and no expansion of ES cell colonies was observed after one month. Similarly, we failed to maintain undifferentiated hES cell colonies on Matrigel after one passage, if purified hES cells or significantly diluted co-cultures (5% or 1:20, so that the "carryover" MEFs were negligible) were seeded (Fig 2). Our data is consistent with a recent publication reporting the similar difficulty in passaging and maintaining hES cell lines in an undifferentiated state using Matrigel supplemented with conditioned medium for >35-42 days (37). Our data together with Richards et al. (37) acknowledged our *current* inability to separate the soluble and cell-associated components required for prolonged growth and expansion of hES cells in culture.

The culture-expanded and highly homogenous hMSCs will enable us to perform detailed analyses, which were previously impossible with heterogeneous cell populations such as (p3) MEFs. A number of reports examined the production of cell adhesion molecules and growth factors/cytokines by MSCs. For example, we have detected both mRNA and protein for LIF as well as IL-6 and IL-11 (26, 28). Indeed, hMSCs fully supported the proliferation of undifferentiated mouse ES cells in the absence of exogenous LIF, either FBS-containing or the hES cell culture medium (unpublished data, August 2002). It is unclear whether MSCs acquired the ability to support the hES cell growth once cultured in the hES cell

culture medium. To this end, we will continue the investigation to separate soluble factors and ECM/cell adhesion molecules made by MSCs.

Richards et al. also reported that fetal skin and muscle cells from 14-week-aborted fetuses can also support prolonged growth of hES cells (37). Although it is an important breakthrough, "ethical concerns regarding the derivation of fetal cells from human abortuses" limit their uses, as noted by the authors (37). They also used human feeder cells derived from adult fallopian tubal (AFT) tissues after hyterectomy. Unless the AFT cells that the authors derived can be immortalized and proliferate indefinitely in culture, the use of primary AFT cells to culture various ES cell lines for ES cell expansion will not be practical. In comparison, hMSCs can be readily derived from adult healthy donors or perspective patients, and expanded million fold before being used in co-culture for supporting hES cell expansion. Once hMSCs prove to support other ES cell lines and/or the derivation of new ES cell lines, this novel serum-free, animal cell-free culture system would provide a clinically and ethically feasible method to vastly expand hES cells at a clinical scale. Additional advantages of using hMSCs in culture to support the hES cell growth include that autologous and unrelated (allogeneic) hMSCs have been tested in clinical transplantation setting, and that unrelated MSCs do not generate alloreactive T lymphocytes in culture and in large animals (31, 38). In fact, recent data even revealed a remarkable ability of hMSCs to down-regulate an allo-immune response of the host to the third party graft (38-39). One can envision that the presence of hMSCs (derived from the patient or from a universal source) may help to induce immune tolerance and reduce the allogenic rejection to the hES cell-derived progeny (a third party graft), when transplanted into a patient whose genotype is different from the hES cells.

Although hMSCs can proliferate in culture for a long time, we often observed that their proliferation rate and differentiation potential were significantly reduced after 6 passages (>25 population doublings). Their ability to support hES cell expansion was also reduced after 6 passages (unpublished data, September 2002). Similarly, we also found that p4-5 MEFs also had reduced activities to support hES cell expansion as compared to p3 MEFs that we used routinely. Thus, we may have to use p2-5 hMSCs to achieve maximal hES cell expansion. With hMSCs, three groups recently reported that the immortalization of their proliferative and primitive potentials by over-expressing the TERT gene, the catalytic subunit of telomerase (40-42). Moreover, Dr. Verfaillie's group reported that the adult BM derived MAPC (multipotent adult progenitor cells) proliferate in culture for an extended time while maintain their pluripotent differentiation potentials (21-24). It is of great interest to determine whether these "immortalized" adult marrow-derived cells can also support hES cell expansion as hMSCs we used in this report.

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Acknowledgement

We thank Gail Stetten, Sarah South, Joseph McMichael in the Laboratory of Prenatal and Research Cytogenetics at the Johns Hopkins Hospital for their expert assistance with cell karyotyping analyses, Drs. Saul Sharkis and Michael Shamblott for critical reading of the manuscript, and Developmental Studies Hybridoma Bank (Iowa City, IA) for SSEA-1 and SSEA-4 antibodies.

Table 1. Expression of selected cell surface markers on culture expanded hMSCs and hES cells (on hMSCs)

Cell Type	APase	SSEA-1	SSEA-4	Endoglin (CD105)	MHC class I	MHC class II	AC133 (CD133)	Thy-1 (CD90)	CD34	PECAM-1 (CD31)	CD45
hMSCs	-	-	-	++	++	-	-	++	-	-	-
hES cells	++	-	++	-	+	-	+	+	-	-	-

-: no difference or <2 fold higher than background; +: 2-10 fold above background; ++: >10 fold higher than background.

Fig 1. Morphology and APase staining of hES cell colonies cultured on hMSCs. hES cells were continuously (for 5-7 passages) cultured on non-irradiated (**A**) or irradiated hMSCs (**B** & **C**). Representative images of live hES cell colonies are shown on non-irradiated and irradiated hMSCs are shown in **A** (10x) and **B** (20x). Individual spindle-shaped fibroblastic hMSCs are also visible in (**A**) and (**B**). After fixing, the co-culture was stained for the APase activity that is primarily on cell surface (**C**). Note that the hES cell colony is positive for APase activity whereas the majority of hMSCs are negative. Bar scales: 100 μm, 10 μm and 5 μm in **A**, **B**, and **C**, respectively.

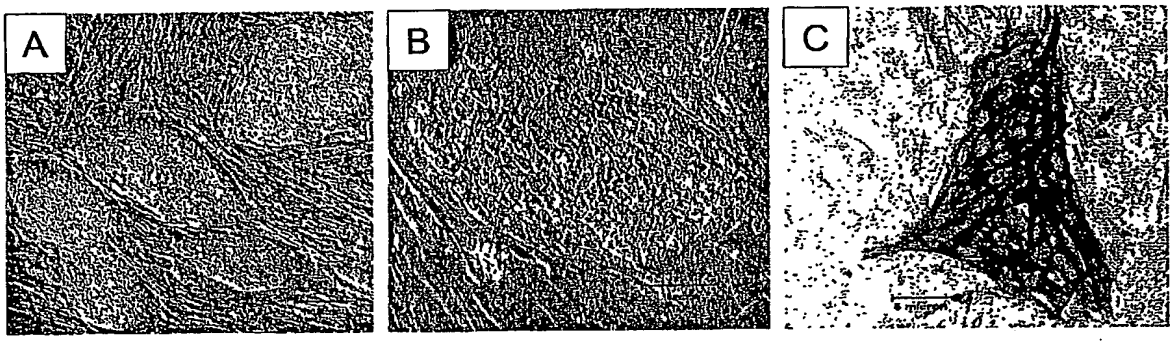
Fig 2. Numbers of ES cell colonies after co-culture with MEFs, hMSCs and Matrigel as adhesion matrices. After expansion on hMSCs for 6 passages, hES cell aliquots (1/20 or 5%) were seeded in 6-well plates containing irradiated MEFs (n=3), hMSCs #1 (n=2), hMSCs #2 (n=3), or coated with Matrigel (n=3) as described in details in "Material and Methods". Six days after, numbers of live ES cell colonies (≥50 cells, as shown in Fig 1) were counted in each well. The mean and standard error of each sample are plotted.

Fig 3. Human ES cells expanded on hMSCs retained the SSEA-4 expression on cell surface. Six days after plating, hES cells/hMSCs co-cultures were fixed and then labeled with diluted hybridoma supernatants recognizing either SSEA-4 (A & B) or SSEA-1 (C & D). Goat anti-mouse IgG conjugated to the Alexa 546 was then added as a red fluorchrome. The nuclei of both hES cells and hMSCs were counter-stained by Hochest 33358 (blue). Micro-photographs of fluorescence (left, A & C) and bright field (right, B & D) images of the same colonies were taken and recorded in Kodak film.

Fig 4. Flow cytomertic analysis of SSEA-4$^+$ cells before and after cell isolation. The hES cells co-cultured on hMSCs were harvested by trypsin/EDTA digestion. The single cell suspension was labeled with the SSEA-4 mAb. The labeled cells were then incubated with magnetic beads conjugated with anti-mouse IgG antibodies. Aliquots of input cells (**A**) and isolated cells after positive selection (**B**) were further incubated with anti-mouse IgG antibodies conjugated with PE (AM-PE). The histograms of the two samples are shown in dark open lines. The profile of a background control (omitting the SSEA-4 primary mAb) is shown as the filled gray line. The percentages of the gated positive cells in the marked region (M1) are also indicated.

Fig 5. RT-PCR analysis of the Oct-4 gene expression in hES cells cultured on MEFs and hMSCs. After co-culture with hMSCs for 5 passages, hES cells were purified from hMSCs by MACS as shown in Fig 4. Similarly hES cells co-cultured on MEFs were also purified. Irradiated MEFs and hMSCs in the absence of hES cells were used as controls. After RT reactions, cDNA was amplified with the primer sets for either the human Oct-4 gene or for the β-actin gene (human and mouse) as a control. Lane 1: hES cells cultured on MEFs; Lane 2: MEFs alone; Lane 3: hES cells cultured on MSCs; Lane 4: MSCs alone.

Fig 6. A normal chromosomal karyotype of hES cells that have been expanded on hMSCs for 9 passages.



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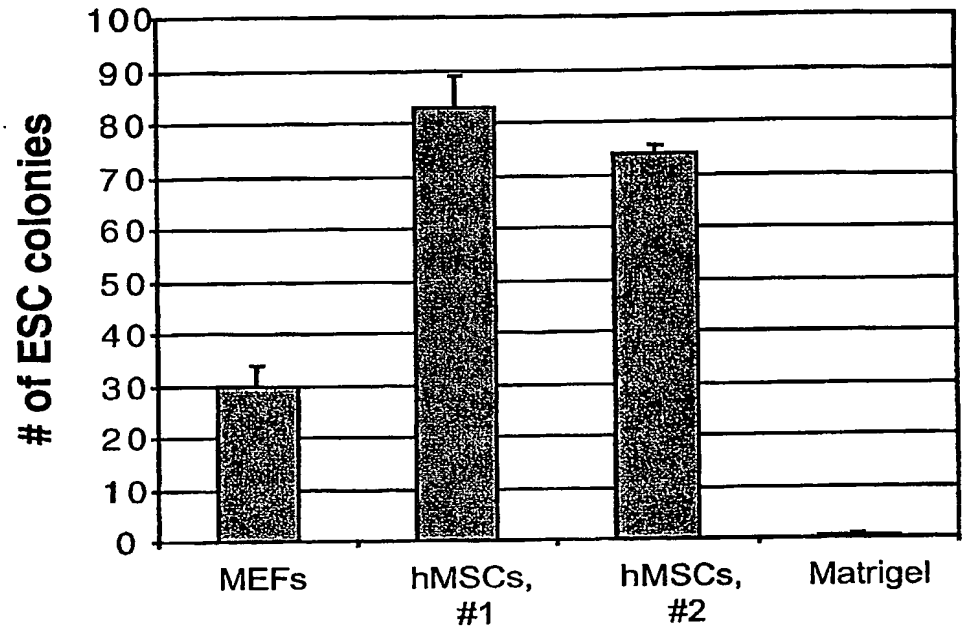

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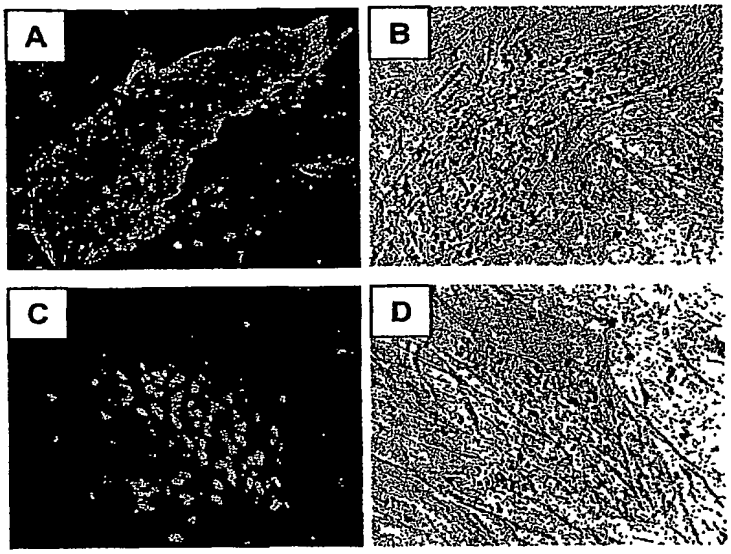

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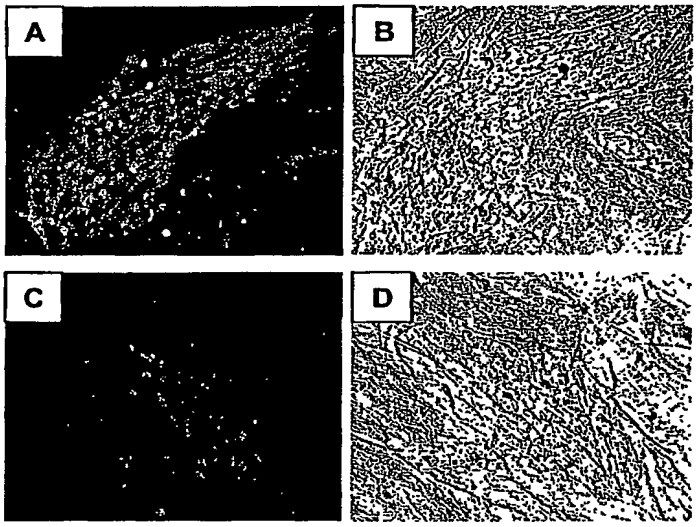

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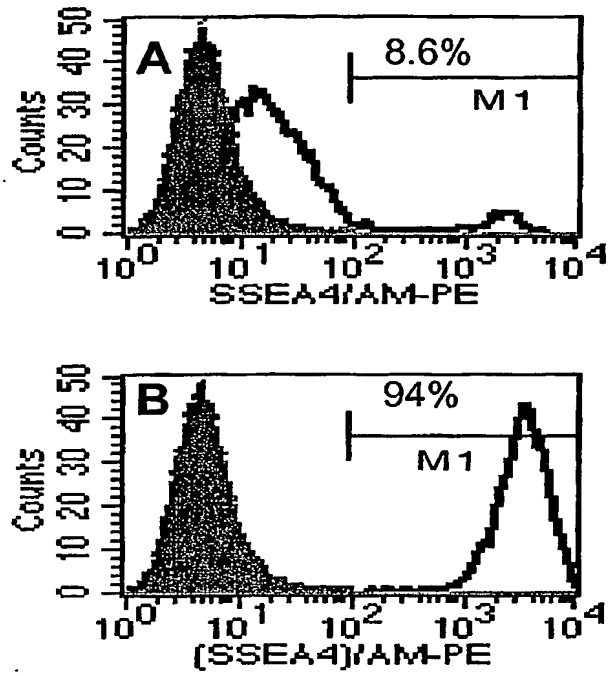

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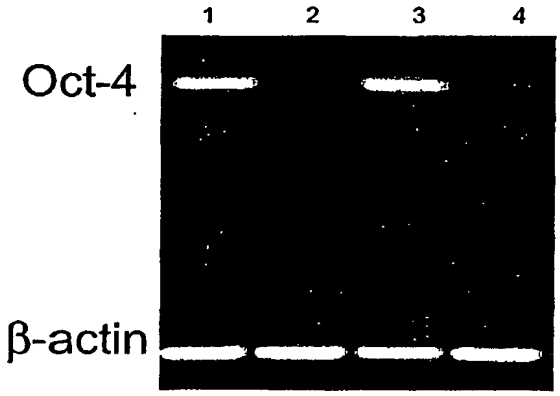

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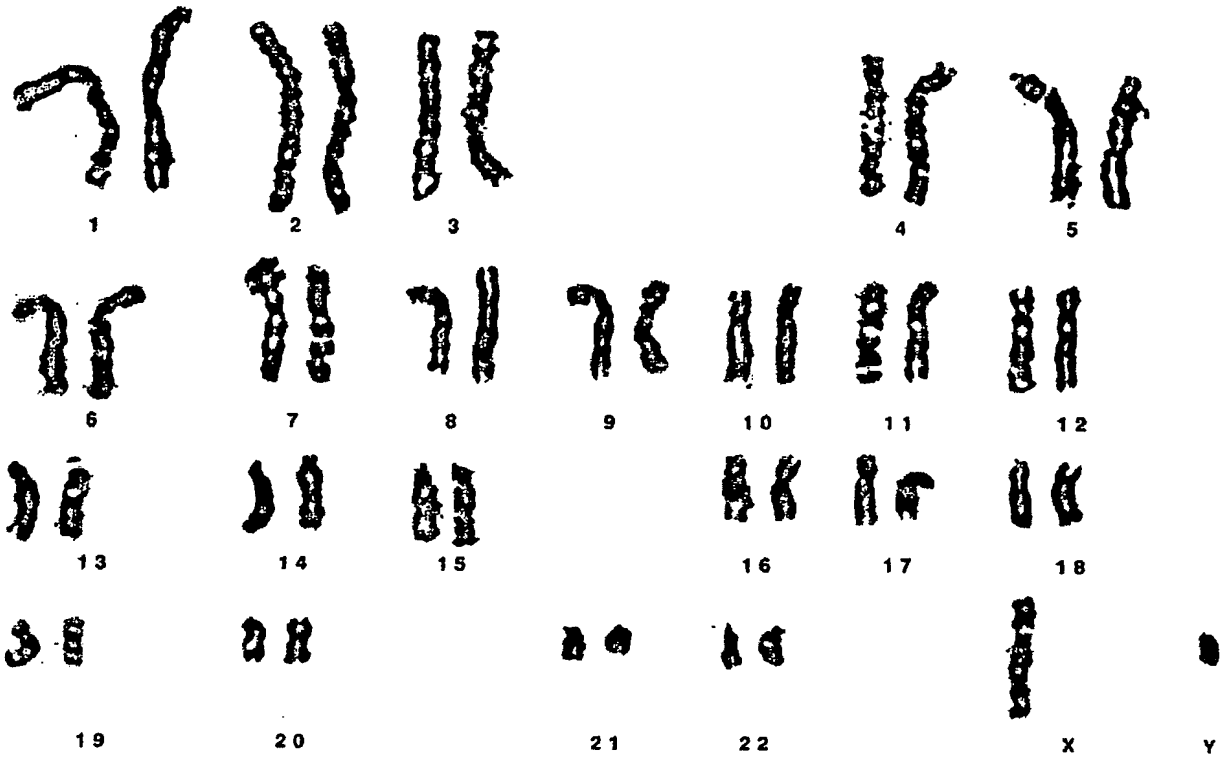

Fig 6. A normal chromosomal karyotype of hES cells that have been expanded on hMSCs for 9 passages.

Johns Hopkins University School of Medicine
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This form is to be completed and submitted to the JHUSOM Office of Technology Licensing (OTL) by anyone who believes they have developed a new invention. The purpose of this form is to enable OTL to evaluate whether legal protection to the invention will be sought and/or commercialization pursued. In order for this Report of Invention to be processed by OTL, it must be signed and dated by all inventors, and by the JHU Department Director for each department involved with the development of this invention. OTL can not process this report until it is complete with all necessary signatures.

INVENTION INFORMATION
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Citizenship: USA Social Security Number:
Department(s) in which invention was developed: Oncology
Are you an HHMI investigator? x No Are you a KKI investigator? x No

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ADDITIONAL INVENTOR(S)

Please copy this page for additional inventors as necessary

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Business phone: Business fax: E-mail:

Business address:

Home phone number: Home fax number: none

Home address:

Citizenship: Social Security Number:

Are you an HHMI investigator? No Are you a KKI investigator? No

INVENTION DESCRIPTION

Describe the invention completely, using the outline given below.

1. **Abstract of the Invention** [Briefly describe the invention]

Expanding human embryonic stem cells (hESCs) efficiently under a clinically applicable culture condition is a prerequisite for their use in a variety of cell therapies and tissue engineering. Currently, Undifferentiated, pluripotent hESCs can be only maintained and expanded when cultured with mouse embryonic fibroblasts (MEFs) or the conditioned medium (CM) from MEFs. This appears the case for all the hESC lines that were mentioned in the published scientific papers. Unfortunately, there are still numerous rodent proteins (or other undefined bioactive molecules) from MEFs or their CM that are in direct contact with hESCs. We report here that we discovered a method to expand hESCs with an equal or better efficiency with the use **of non-transformed, normal human cells derived from adult bone marrow (BM)**. These BM-derived human cells can be readily derived from adults with a routine procedure, and expanded >1 million fold before being used for support of hESC expansion. Undifferentiated hESCs that were co-cultured with these BM-derived feeder cells amplified >100 fold during the 30 day continuous culture. The amplified hES cells displayed unique morphology and molecular markers, characteristic of undifferentiated hESCs. The amplified hESCs can be also easily isolated from the feeder cells (that have been irradiated, and unable to proliferate) during harvest. This novel culture system provides a clinically and ethically feasible method to vastly expand hESCs.

2. **Problem Solved** [Describe the problem solved by this invention]

Currently, maintenance and proliferation of hESCs require mouse embryonic fibroblasts (MEFs) as "feeder" cells (1-4). Unlike mouse ESCs, the maintenance of hESCs could not be achieved by adding known growth factor such as LIF in the absence of MEFs (1-4). Other factor(s) made by MEF are also required. It was recently reported by Geron scientists that MEFs are not absolutely required. However, hESCs have to be placed on matrigel (a basement membrane extract from mouse NHS tumors) and cultured with the CM (containing secreted molecules) collected from cultured MEFs (4). Unfortunately, there are still numerous rodent proteins (or other undefined bioactive molecules) from either MEFs or matrigel that are in direct contact with hESCs. It is highly desirable to avoid rodent feeders cells and potential pathogens carried in these cells, CM and crude extracts (4-5).
Therefore scientists have been looking for human cells that can support the growth of undifferentiated hESCs. No success was reported in scientific papers (last searched, 4/15/2002). In an interview with the *Science* magazine, an Israeli group said they can grow with hESCs with "human fetal cells" as feeders (5). While others look for a replacement of MEFs with human embryonic/fetal cells, we attempted to use human adult cells.
We reported here that we discovered a method to expand hESCs with an equal or better efficiency with the use of non-transformed, normal human cells derived from adult bone marrow (BM). These BM cells can be readily derived from adult patients or healthy individuals, and expanded million fold before being used for support hESC expansion. This novel culture system provides a clinically and ethically feasible method to vastly expand hESCs, a prerequisite for any clinical uses.

3. **Novelty** [Identify those elements of the invention that are new when compared to the current state of the art]

We established a method to derive and expand human cells (from adults) that can fully substitute MEFs in support of the growth of undifferentiated hESCs. The type of human cells we tried so far is from bone marrow aspirate from adults (after a minor surgery). Adherent, fibroblastic marrow stromal cells (MSCs) were expanded selectively in culture. After 2 passages in culture, nearly homogenous hMSCs are obtained after an expansion of a thousand fold. These culture-expanded hMSCs that function as multipotent stem cells (MSCs) can generate multiple types of cells (such as those found in bone or connective tissues) when induced to differentiation under specific conditions, and support hematopoiesis from hematopoietic stem cells. The major discovery we made (novelty) is that MSCs can also support the growth of un-differentiated hESCs under the culture condition we devised. Therefore, we invented a clinically and ethically feasible method to vastly expand hESCs for treating human diseases, using adult human cells as a source of feeder cells or missing growth factors.

4. Detailed Description of the invention:

On a separate page(s), attach a detailed description of how to make and use the invention. The description must contain sufficient detail so that one skilled in the same discipline could reproduce the invention. Include the following as necessary:

1- data pertaining to the invention;
2- drawings or photographs illustrating the invention;
3- structural formulae if a chemical;
4- procedural steps if a process
5- a description of any prototype or working model;

In general, a manuscript that has been prepared for submission to a journal will satisfy this requirement.
Attached: Key figures; An incomplete draft of manuscript
(4 pages) (To be Sent)

Linzhao Cheng.

5. Workable Extent/Scope [Describe the future course of related work, and possible variations of the present invention in terms of the broadest scope expected to be operable; if a ***compound,*** describe substitutions, breadth of substituents, derivatives, salts etc., if ***DNA or other biological material,*** describe modifications that are expected to be operable, if a ***machine or device***, describe operational parameters of the device or a component thereof, including alternative structures for performing the various functions of the machine or device]

1. **A cell-free system using conditioned medium (CM) from hMSCs**
   Ongoing experiments are aimed to determine whether secreted molecules in the hMSC CM can replace live MSCs to support the growth of hESCs. In analogy to a previously published study using the CM from MEFs (4), we may need to add extra-cellular matrix (ECM) molecules such as matrigel as attachment factors in addition to the CM of hMSCs. Our preliminary data are promising, although we have not finished the characterization of the expanded hESCs in the cell-free culture system using the CM of hMSCs. Similarly, we will test whether defined and purified ECM molecules such as laminin can substitute Matrigel, a crude extract from mouse tumors. It is likely that we will have a cell-free system using the CM of hMSCs and (human) laminin, completely devoid of rodent (or any animal) cells or derived molecules.

2. **Identify secreted growth factors made by hMSCs and acting on hESCs**
   If the CM from hMSCs (together with ECM molecules) is sufficient to support the growth of undifferentiated hESCs, then we have set up a system to identify the elusive human growth factor that stimulate the growth of hESCs. Using the standard biochemical and genetic (such as genechips) approaches, it is relatively straightforward to identify such a growth factor, which are made by hMSCs and encode secreted molecules acting on hESCs. The growth factor may be completely novel or known before (acting in other cell systems). Once such a growth factor is identified, we will be able to synthesize a large quantity by standard recombinant DNA technology (like recombinant EPO or insulin production). In turn, we will be able to establish a cell-free, animal protein-free, precisely-defined and scalable culture system to vastly and efficiently expand hMSCs.

3. **The identified growth factor may also act on other stem cells isolate from adult tissues**
   The same molecule acting on hESCs may also help the self-renewal and "trans-differentiation" of adult stem cells residing in a various adult tissues. In contrast to hESCs, these adult stem cells are normally limited by developmental potentials and difficult to expand in culture without differentiation. The above identified growth factor of hESCs may also potentiate or enhance the proliferation of adult stem cells that are rare in numbers and limited in potentials. In addition to being used as above for *ex vivo* (in culture) expansion of adult stem cells, we may be able to expand adult stem cells *in vivo* by direct injection the growth factor into appropriate sites in patients.

4. **Elevate the level of this growth factor expression by gene delivery or specific drugs**
   a. gene therapy approach;
   b. identify small-molecule drugs that elevate the expression of the gene encoding the growth factor.

6. References [Please list the closest and most relevant journal citations, patents, general knowledge or other public information related to the invention]

1. Thompson JA et al. (1998). Science, 282:1145;
2. Thomson JA. US Patents 5, 843,780 and 6,200,806; US Patent Application 09/522,030.
3. Reubinoff BE et al. (2000). Nature Biotechnology, 19:399.
4. Xu C et al. (2001). Nature Biotechnology 19:971.
5. A report in *Science*, March 8, 2002 (295:1819).

RESEARCH SUPPORT INFORMATION

Indicate ALL contributions to the development of the invention in terms of personnel, money, materials and facilities etc.
The development of the invention was done by a JHU faculty at JHU facilities. The Cheng members (research associates and assistants) provided necessary technical assistance.

Check each funding source that applies to this invention:

Federal Sponsor(s) and √ Other

For each funding source, provide the following information:

Granting/Funding Source Award/Contract Number Title of Grant

The WW Smith Charitable Trust. #C9902. "A candidate cytokine gene specifically expressed in blood stem cells". Used to purchase the H1 hESC line from WiCell.
NCI/NIH. P30 CA 06973. "Cancer Center Support Grant". Research on hMSCs and hESCs

Were any materials under a Materials Transfer Agreement used? yes

If yes, please provide the following information for each material and attach a copy of the MTA:

Source of Materials	Materials
WiCell (U. of Wisconsin) (the H1 hESC line is included in the NIH list) (MOU: Johns Hopkins 02-W017; SLA: 02-W018) Copies enclosed	The H1 hESC line

SIGNATURE PAGE and ASSIGNMENT OF INVENTION

In order for this Report of Invention to be complete and processed by OTL, it must be signed and dated by:

(1) all inventors, and

(2) by the JHU Department Director for each department involved with the development of this invention as indicated on page one of this form.

I/we, the Inventors, hereby certify that the information set forth in this Report of Invention is true and complete to the best of my/our knowledge.

I/we, the Inventors who are subject to The Johns Hopkins University Intellectual Property Policy and are not under an obligation to assign intellectual property rights to another party[1], hereby affirm that in consideration for The Johns Hopkins University's evaluation of commercial potential and a share of income which I/we may receive upon commercialization of my/our invention, I/we on the date of my/our signature as indicated below do hereby assign and transfer my/our entire right, title and interest in and to the invention described herein unto The Johns Hopkins University, its successors, legal representatives and assigns.

[1]HHMI and KKI Inventors are subject to a separate assignment.

Inventor Signature	Linzhao Cheng Typed or Printed Name	Date
Department Director Signature	Martin Abeloff Typed or Printed Name	Date

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Phone: (410) 347-3216 Fax: (410) 347-3201

Fig. 1

Expanding hESCs on human marrow stromal cells (hMSCs) from adult bone marrow

hES cells have been cultured on hMSCs and expanded >100 fold. During the 30 days of continuous culture, hES together with MSCs were passaged 4 times (splitting 1:8, 1:4, 1:3 and 1:3, respectively).

Unique morphology of undifferentiated ES cells;

The expanded cells expressed Alkaline phosphatase (APase) and SSEA-4 but not SSEA-1, characteristic markers of human ES cells.

The expanded cells expressed a high level of Oct-4, a transcription factor unique to undifferentiated stem cells.

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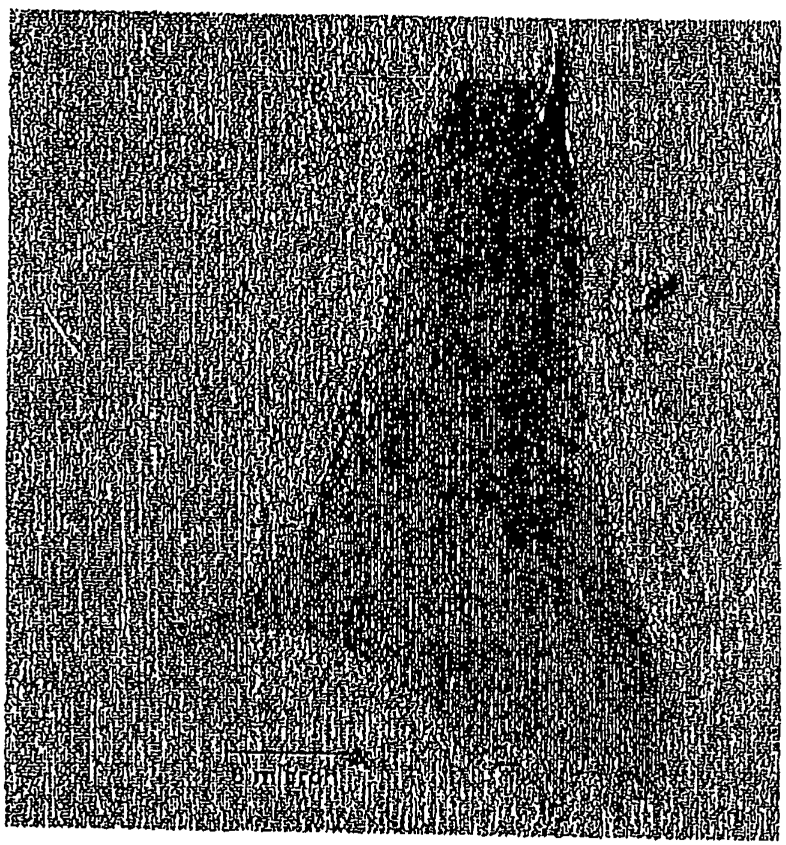
Fig. 2

hES cells cultured with hMSCs expressed markers unique to undifferentiated ES cells



APase (primarily on cell surface) staining with BCIP/NBT.

Note: hMSCs (undifferentiated) are "-" for APase.

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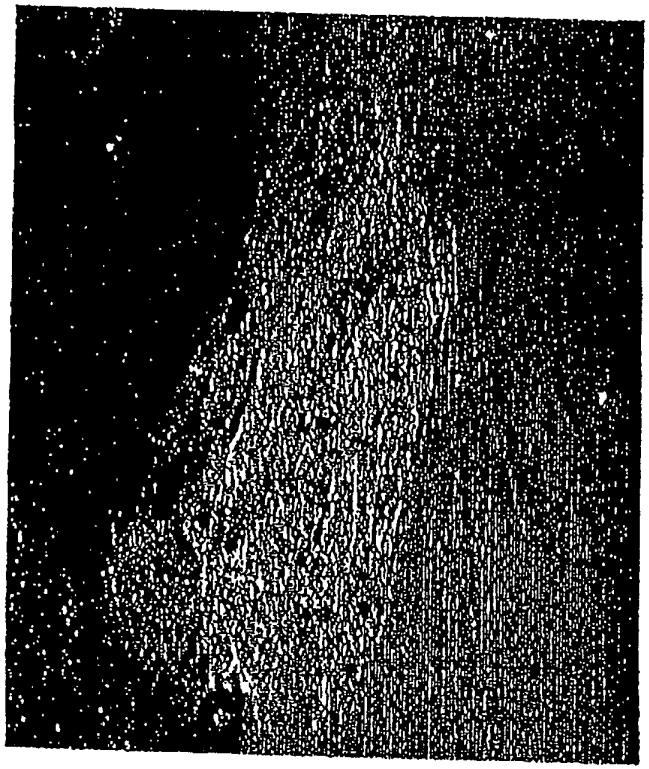
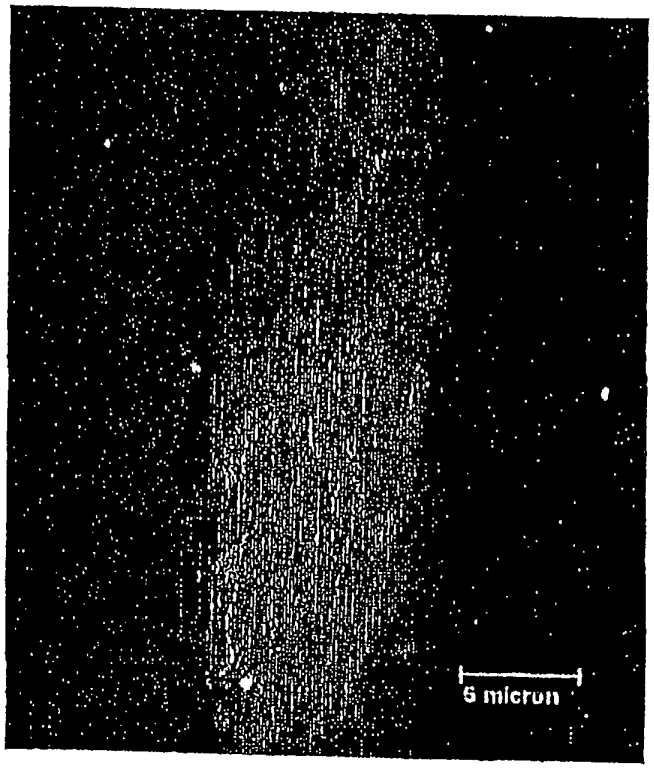
Fig. 3

hES cells cultured with hMSCs expressed SSEA-4, unique to undifferentiated ES cells



Composed bright field and fluorescent images



Fluorescence of SSEA-4 (cell surface) staining

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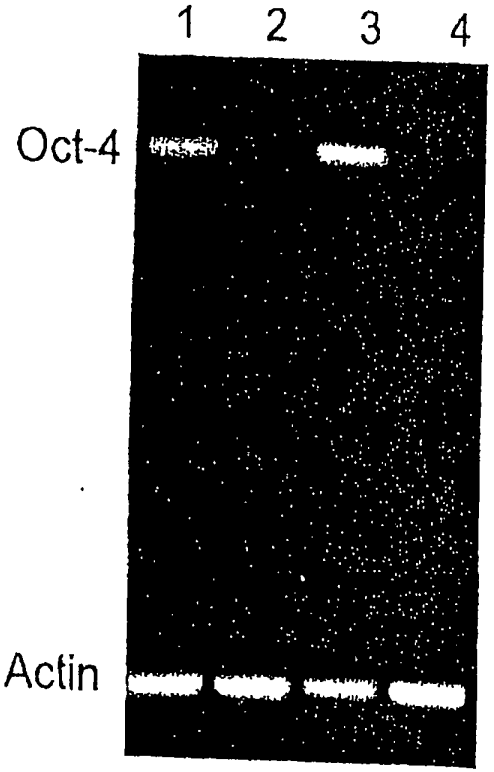
Fig. 4.

Human ESCs cultured with hMSCs expressed Oct-4, unique to undifferentiated stem cells

RT-PCR products



1. Isolated hMSCs (cultured with MEFs): ++
2. MEFs: -
3. Isolated hMSCs (cultured with hMSCs): +++
4. hMSCs: +

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Growth of hESCs supported by adult human marrow cells

Xiangcan Zhan, Holly Hammond, Zhaohui Ye and Linzhao Cheng

Johns Hopkins Medical School

Expanding human embryonic stem cells (hESCs) efficiently under a clinically applicable culture condition is a prerequisite for their use in a variety of cell therapies and tissue engineering. Unlike mouse ES cells, adding growth factors such as LIF is insufficient to maintain undifferentiated hESCs in the absence of feeder cells. Currently, undifferentiated and pluripotent hESCs can be only maintained and expanded when cultured with mouse embryonic fibroblasts (MEFs) or the conditioned medium from MEFs. We know little about the hESC-required growth factors made by MEFs. While others look for a replacement of MEFs with human embryonic/fetal cells, we attempted to use human adult cells.

We report here that we discovered a method to expand hESCs with an equal or better efficiency with the use **of non-transformed, normal human cells derived from adult bone marrow (BM)**. We devised an improved method to expand human marrow stromal cells (hMSCs) >1 million fold and used hMSCs to support hESC growth. Undifferentiated hESCs that were co-cultured with the hMSC feeder were amplified >100 fold during the 30 day continuous culture. The amplified hES cells displayed unique morphology and molecular markers, characteristic of undifferentiated hESCs. This novel culture system provides a clinically and ethically feasible method to vastly expand hESCs for treating human diseases, and an assay system to identify growth factors that are made by hMSCs and required by hESCs.

Introduction

Several distinct types of stem cells have been isolated from adult bone marrow. Adult marrow is the primary site to isolate hematopoietic stem cells (HSCs), the common precursor of blood

and immune cells (as well as osteoclasts, xxx). A multipotent non-hematopoietic stem cells have been isolated from adult human and animal bone marrow. It was also reported in recently years that highly purified marrow cells can differentiated into many other types of cells such as hepatocytes in liver, neurons and glial cells in brain, satellite cells in skeletal muscles and cardiomycytes in hearts. It remained to be determined whether a single adult marrow cell contains all these developmental potential ("trans-differentiation" or "plasticity") or multiple cells contributes observed distinct activities. However, it is clear that adult marrow contains cells and microenvironment supporting stem cell's potential to generate a variety of specialized cells found in embryos and adults. Therefore we focused on adult human marrow cells, esp. MSCs, to investigate whether we can replace MEF to support hES cell proliferation.

In recent years, several groups have developed improved methods to obtain large numbers of marrow stromal fibroblasts in culture from adult human marrow, either by physical isolation of precursor cells followed by culture expansion, or by direct culture selection and amplification. These marrow fibroblastic cells have be termed as either stromal progenitor cells (SPCs) reflecting their proliferation potential in culture (Simmons), marrow stromal cells (MSCs) reflecting the source and method of the derivation (Prockop, 1997), or mesenchymal stem cells (MSC) reflecting developmental potentials of primary and culture-expanded cells (Caplan, 1991; Pittenger et al., 1999; Cheng et al, 2000). The latter two methods, which are essentially the same and in essence similar to a historic paper, are widely used by many investigators. The derived marrow (fibroblastic) stromal cells that are function as non-hematopoietic multipotent stem cells were collectively called here as MSCs. After two passages (approximately 14 cell divisions) in a selective medium supplemented with fetal bovine serum, culture-expanded human MSC are morphologically and phenotypically homogenous and essentially free of macrophage or

adipocyte contamination (Pittenger et al., 1999; Cheng, 2000). Since then, we have further simplified derivation and culture of MSCs from adult human bone marrow. BM mononuclear cells after Ficoll density selection (from either CD34+ or CD34- fractions) can also generate MSCs. Addition of bFGF (1 ng/ml) provides a consistently optimal growth condition and essentially alleviates the need to screen favorable bovine serum lots.

These highly homogenous and cultured expanded MSC enable us to perform detailed analyses which were previously impossible with mixed "stromal" cell populations. In addition, the expanded adherent cells provide a feasible source of feeder cells to support HSCs or ESCs. The culture-expanded MSCs supported $CD34^+$ HSCs in long-term culture assays and their differentiation into erythroid, myeloid, megakaryocytic or osteoclastic or B cell lineages in the absence of added cytokines (1998; 2000). The activity is due to, at least in part, that the these culture-expanded cells express various hematopoietic cytokines including LIF, IL-6, IL-11 as well as SCF, and Flt3/Flk2 ligand (FL) (Cheng 2000). These led us to investigate whether MSCs derived from adult BM can also support the growth of hESCs.

We report here that culture-expanded MSCs can replace MEF and fully support growth of undifferentiated hESCs. After co-culture with hMSCs (mitotically active or inactive), hES cells expanded >100 fold during the 30 day continuous culture. The expanded cells retained unique hESC morphology and expressed markers such as APase and SSEA4, characteristic of undifferentiated hESCs.

Results

We obtained the H1 hESC line (p20) from WiCell and expanded the stock in co-culture with irradiated MEFs as instructed. We continuously cultured for 3 months and split on average once

a week. We found that numbers of hESC colonies expanded 1-2 fold each passage, as described in the instruction from the provider. Later we attempted to improve split and plating efficiencies by using different cell dissociation methods. By using an optimal trypsin/EDTA solution to harvest cells followed by the addition of a trypsin inhibitor, we managed to increase plating efficiencies to 5-6 fold per passage. The new dissociation method also allowed us to make single cell suspensions more efficiently. This in turn allows the selection of undifferentiated hESCs based on SSEA-4 antigen expression on cell surface, using FACS or MACS. Periodically we selected SSEA-4+ undifferentiated hESCs cells and re-plated them onto irradiated MEF for one passage before critical experiments. The purity ranges from 65% to 99% (analyzed by FACS following MACS isolation).

1. hESCs grew well on the feeder cells of irradiated hMSCs. Fig 1

   So far we cultured for 5 passages (a total of 35 days)

   Good morphology (Fig 2)

2. The expanded hESCs co-cultured with hMSCs retained molecules markers unique to undifferentiated hESCs.

   After 3 or more passages with hMSCs, the expanded hESCs were analyzed by the expression of markers such as APase and SSEA-4. By histochemical staining, hESCs colonies are APase positive, while the MSCs feeder cells are largely negative (Fig 1b). IN hESCs, APase activities were preferentially expressed on cell membrane. Like irradiated MEFs, few single MSCs with apoptotic morphology were stained weakly for APase. This is consistent with the report that undifferentiated hMSCs are APase negative until induced to differentiate to osteoblasts.

   The expanded hMSCs were also stained with 2 glycolipid cell surface antigens, SSEA-4 and

SSEA-1. While SSEA-4 was positive on hESCs (1), SSEA-1 is positive in mouse ESCs but negative for hESCs. The co-culture of hESCs with irradiated hMSCs (p3) was stained in parallel with mouse mAb against either antigen. A high level of SSEA-4 expression was found in expanded hESCs while absent in the hMSC feeder. The SSEA-1 was undetected in either the expanded hESCs or hMSCs (Fig 2), but positive in mouse ES cells or human CD15+ granulocytes (not shown).

Discussion

We discovered a method to expand hESCs with an equal or better efficiency with the use of non-transformed, normal human cells derived from adult bone marrow (BM). These BM cells can be readily derived from adult patients or healthy individuals, and expanded million fold before being used for support hESC expansion. This novel culture system provides a clinically and ethically feasible method to vastly expand hESCs for treating human diseases.

Materials and Methods

Human ESCs. The H1 ESC line was obtained from WiCell Research Institute, Inc., and initially cultured as instructed by the provider. MEFs (purchased from Speciality Media, Inc) were used as feeder cells for the hESCs. 25,000 irradiated (5,000 rads) MEFs were plated per well (9.4 cm2) in 6-well plates. The hESC culture medium is based on the KO-medium plus 20% of KO serum replacement (Life Tech.), 50 um b-Mercaptoethanol, 2 mM Glutamine and 4 ng/ml bFGF (the complete hESC medium). Cell cultures were incubated at 37°C in 5% CO_2 in air and 95% humidity (the standard culture condition). Once hESC colonies grew maximal sizes (6-10 days after seeding), cells in the co-culture were digested by 1 ml of 0.05% trpsin/0.53 mM EDTA for 5 min. The digestion was stopped by adding 0.6 ml of trypsin inhibitor (0.5 mg/ml). The dissociated cells were split from 1:1 to 1:50 and seeded onto feeder cells or Matrigel-coated plates.

Human MSC Isolation and expansion. Bone marrow samples collected from healthy and consented human donors were purchased from AllCells company (San Mateo, CA). Heparinized bone marrow was mixed with equal volume of phosphate buffered saline (PBS) (Life Technologies, Gaithersburg, MD) and centrifuged at 900 x g for 10 minutes at 25°C. Mononuclear cells (MNCs) were isolated by the standard density (1.077 g/ml) centrifugation using Ficoll (Pharmacia, Piscataway, NJ). As compared to a previous protocol using Percoll (1.073 g/ml, also from Pharmacia), the Ficoll method yielded 2 fold more total MNCs but the same amounts of MSCs (see below). MNCs at the interface were recovered, diluted with 5 volumes of PBS, recovered by centrifugation and finally resuspended in hMSC medium composed of Dulbecco's Modified Eagles Medium (DMEM) with low glucose (DMEM-LG) (Life Technologies), 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT, Life

Technologies or Gemini), 1% antibiotic-antimycotic stock solution (Life Technologies) and 1 ng/ml bFGF (Life Tech or Peproetch). The addition of bFGF to the "generic" gave consistent and optimal growth with different batches of FBS from various suppliers. The complete hMSC medium (with 1 ng/ml bFGF) performed slightly better than the MSCGM we purchase from Biowittaker in one head-to-head test. Cells were plated into 185 cm^2 flasks (Nunc) at a density of $3x10^7$ MNC/flask and the cultures were incubated at 37°C in 5% CO_2 in air and 95% humidity. The medium was exchanged after 48 hours and every 3-4 days thereafter. When the cultures reached approximately 90% of confluence, hMSCs were recovered by the addition of 0.025% trypsin/0.53 mM EDTA solution (Life Technologies) and re-plated into passage culture at a density of 5,000 to 10,000 cells per cm^2. For the colony formation assay, aliquots (up to 1.6 $x10^6$ cells/well) of MNCs were plated into 6-well culture dishes for 14 days with the complete MSC medium. Then they were washed, fixed with 4% formalin solution (Fisher) and stained with 0.1% solution of crystal violet (Sigma) for 30 minutes. Fibroblastic MSC colonies were found in MNCs from bone marrow (approximately one in 20,000 MNCs after Ficoll or one in 10,000 MNCs after Percoll), but not from cord blood or peripheral blood with or without the G-CSF mobilization.

Co-culture of MSCs with ESCs Culture expanded hMSCs after 2 passages (P0 and P1) or more passages (up to P5 tested) were treated similarly as MEFs for feeder cell preparations. Briefly, MSCs were plated at ≥2,600 cells per cm^2 (25,000 cell per well in 6 well plates) with or without gamma irradiation (5,000 rads). Before plating hESCs, MSCs cultured with the serum-containing complete medium were washed by PBS and the KO-medium (Life Tech). The ESCs/MSCs co-culture were incubated and split as the ESCs/MEFs co-culture described above.

Immuno-fluorescence and APase staining. Co-cultures used for immuno-fluorescence analysis were established in either 6-well or 24-well plates. Prior to this analysis, adherent cell layers were fixed by the addition of 10% formalin (15 minutes). The fixed cells were incubated 15 minutes with a 2% NGS (normal goat serum) solution. The co-cultures were stained with diluted (1:100) hybridoma supernatants recognizing either SSEA-4 or SSEA-1 antigen. After incubation in the dark for 1 hour at RT or overnight at 40C, fixed cells were washed extensively before the secondary staining reagent were added. Goat anti-mouse IgG conjugated to the fluorochrome Alexa 568 (Molecular Probes, Portland, Oregon) was added for 45 minutes at RT. The nuclei of hESCs (Positive for SSEA-4 but negative for SSEA-1) and hMSCs (negative for either antigen) were count-staining by Hochest 33358 (Molecular Probes). Immunofluorescence analysis was performed with a Nikon microscope with separate filters for either Hochest (blue) or red fluorescence, or a triple filter for blue, green and red fluorescence simultaneously.

Flow cytometric analysis. Cells were harvested from co-cultures by gentle digestion with 0.025% Trypsin/0.53 mM EDTA solution, washed once in FACS buffer (2% BSA, 2 mmol/L EDTA and 0.1% Azide in PBS) and suspended in 100 ul FACS buffer containing 2 mg/ml human IgG (to block non-specific IgG binding). Two μg/ml of APC-conjugated anti-CD34 antibody (Clone HPCA-2, mouse IgG1) from Becton Dickinson (Mountain View, CA) and 2 μg/ml R-PE conjugated anti-CD41a antibody (PharMingen) were added. The mixtures were incubated on ice for 30 minutes. Cells were then washed once in the FACS buffer and resuspended in 0.4 ml of FACS buffer containing PI. The appropriate conjugated, mouse IgG1 control antibodies were used to establish non-specific staining. Non-viable cells containing PI were excluded from cell analysis. A FACS Calibur flow cytometer (Becton Dickinson), equipped

with a 15 mW Argon-ion laser, was used for these analyses. Selected events (1 X 10^4) were collected for each sample and analyzed using the CellQuest software (Becton Dickinson).

RNA preparation and RT-PCR Analysis. Total RNA was extracted from monolayers of culture-expanded hMSCs (passage 2-3)

Reverse transcriptase (RT)-coupled polymerase chain reaction (RT-PCR) was performed using the RNA PCR Kit (Perkin Elmer, Foster City, CA), according to the manufacturer's instructions. Total RNA (0.5-1.0 μg) was used as a template for the RT-PCR assay and the RT-PCR products were visualized by ethidium bromide staining, following electrophoresis through a 2% agarose gel.

The sequences (5' to 3') of oligonucleotide primer pairs (Operon, Alameda, CA) used in RT-PCR analyses are listed below. TPO 5' primer: CCTCCTTGGGGCCCTGCAGAGCCT and 3' primer: GGGTGGAGCTGGACCACAGGG (amplified product = 606 bp); IL-2 5' primer: ATGTACAGGATGCAACTCCTGTCTT and 3' primer: GTCAGTGTTG AGATGATGCTTTGAC (amplified product = 458 bp); IL-6 5' primer: GTAGCCGCCCCACACAGACAGCC; and 3' primer: GCCATCTTTGGAAGGTTCAGG (amplified product = 628 bp); SCF 5' primer: CCTCTCGTCAAAACTGAAGGG; and 3' primer: AGGAGTAAAGAGCCTGGGTTC (amplified product = 346 bp); Flt3/flk2 ligand (FL) 5' primer: TGGAGCCCAACAACCTATCTC and 3' primer: GGGCTGAAAGGCACATTTGGT (amplified product = 333 bp). RT was omitted in one set of PCR reactions to confirm that DNA products were exclusively derived from mRNA and not genomic DNA.

The references cited herein are incorporated by reference in their entirety.

We Claim:

1. A method of expanding human marrow stromal cells (hMSCs) as described herein.
2. A method of expanding human embryonic stem cells (hESCs) as described herein.
3. A method of expanding human embryonic stem cells (hESCs) by culturing said hESCs in the presence of human adult bone marrow cells.
4. A population of cells prepared according to any method described herein.
5. A population of cells prepared according to any of claims 1-3.
6. A method of treating a disease by administering to a patient suffering therefrom cells according to any of claims 4-5.

Abstract (244 words)

Prolonged propagation of human embryonic stem (ES) cells is currently achieved by co-culture with primary mouse embryonic fibroblasts (MEFs) serving as feeder cells. Currently, undifferentiated human ES cells could not be expanded in culture in the absence of feeder cells like MEFs. However, the presence of uncharacterized rodent cells imposes a risk to clinical applications of human ES cells. While others looked for a replacement of MEFs with human fetal cells, we attempted to use easily accessible adult cells such as human marrow stromal cells (hMSCs). Culture-expanded hMSCs of passage 2 to passage 5 from multiple donors were used to support the growth of the H1 human ES cell line under a serum-free condition, and they gave similar results. Human ES cell colonies cultured on irradiated hMSC feeders amplified >100 fold during the 30 day continuous culture (in 5 passages), and displayed the unique morphology and molecular markers characteristic of undifferentiated hES cells as observed when they were cultured on MEFs. They expressed the transcription factor Oct-4, a membrane alkaline phosphatase and the SSEA-4 (but not the SSEA-1) marker. Expanded hES cells on hMSCs retained a normal diploid karyotype after 9 passages (>60 days). The well-studied hMSCs (and this animal cell- and serum-free system) may provide a clinically and ethically feasible method to expand human ES cells for novel cell therapies. In addition, this system will help us to identify which cytokines and adhesion molecules are required for the self-renewal of human ES cells.